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# Pheromonal Interactions Among Cordate Gametophytes of the Lady Fern, *Athyrium filix-femina*

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ABSTRACT.—Pheromonal interactions between cordate gametophytes of the lady fern, A. filixfemina, were assayed using a protocol typically used for detecting water-soluble pheromones such as antheridiogen. Three week-old, cordate gametophytes were transferred from multispore cultures grown on nutrient agar to agar containing extracts from a previous generation of gametophytes (treatment) and to fresh nutrient agar (control). Three weeks after transfer, fifty gametophytes were examined from treatment and control plates. Each gametophyte was measured for size (area) and shape (circularity) and scored for number of antheridia and archegonia. Treatment gametophytes were significantly smaller, less circular, had fewer archegonia, and possessed antheridia more often than control gametophytes, a pattern consistent with known antheridiogen effects on gametophytes of transitional morphology and sensitivity. The experiment was repeated using gametophytes that were six weeks old at time of transfer to treatment and control plates. Treatment gametophytes in the second experiment did not differ significantly in size (area) or length from control gametophytes; however treatment gametophytes were more circular and possessed greater widths and length : width ratios, deeper notches, and fewer archegonia. We present a model in which one or more phytochemicals released by cordate gametophytes increase rates of anticlinal division in the apical meristem. The possibilities that the substances involved are phytohormones involved in the development of a notch meristem and cordate morphology in the source gametophyte, and that antheridiogen may be involved, are explored.

Ferns are not as phytochemically diverse as seed plants (Cooper-Driver, 1985). Nevertheless, the ability of many ferns to interact phytochemically with neighboring plants has been well established. The sporophytes of some species, notably *Dennstaedtia punctilobula* and *Thelypteris normalis*, produce allelopaths that suppress germination and growth of gametophytes of the same or other species of ferns (Munther and Fairbrothers, 1980; Raghavan, 1989; Wagner and Long, 1991) or the germination and growth in neighboring seed plants (Horsley, 1977, 1986; Davidonis and Ruddat, 1974; Lyon and Sharpe, 1996).

The most extensively documented phytochemical interactions among gametophytes involve antheridiogen. Antheridiogen is a water-soluble pheromone produced by cordate gametophytes that induces dark germination, precocious maleness, and subsequently retards growth and morphological development in less-developed, acordate neighbors. First observed by Döpp (1950), antheridiogen has been subsequently documented in numerous families of filicalean ferns (Raghavan, 1989; Chiou and Farrar 1997). Along with genetic load, antheridiogen has been suggested as a mechanism promoting relatively

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high rates of cross-fertilization in a group of plants that, given their potentially bisexual gametophytes, appear predisposed to selfing (Soltis and Soltis, 1987; Schneller et al. 1990).

Allelopathic interactions among fern gametophytes have also been reported in which gametophyte development was retarded in crowded conditions relative to less crowded conditions (Smith and Rogan, 1970) or cordate gametophytes in particular retarded spore germination, growth, and survival in acordate neighbors (Bell, 1958; Bell and Klikoff, 1979; Peterson and Fairbrothers, 1980). In these studies, and those regarding antheridiogen, the possibility of phytochemical interactions between cordate gametophytes has remained unexplored. Cordate gametophytes that can perceive the presence of other cordate gametophytes could enhance their own fitness by accelerating their own growth rate, morphological development, or production of archegonia. Such interactions may be particularly relevant to understanding gametophyte ecology and the evolution of phytochemical interactions in ferns. We report the results of a bioassay for water-soluble phytochemical interactions between cordate gametophytes of Athyrium filix-femina var. asplenoides, a species with a previously documented antheridiogen system (Schneller, 1979). In isolation, gametophytes of A. filix-femina remain asexual until they develop a cordate morphology, when they produce archegonia (Schneller 1979). In multispore populations, acordate gametophytes often become male in response to native antheridiogen and both male and female gametophytes are capable of becoming hermaphrodites following prolonged growth (Schneller, 1979). Thus, A. filix-femina possesses a category B pattern of gender expression (Klekowski and Lloyd, 1968).

## MATERIALS AND METHODS

To collect water-soluble pheromones, spores of A. filix-femina were collected in the summer of 1996 from Kanawha State Forest in Charleston, WV, and stored in glass vials at room temperature. The spores were then surface sterilized (Dyer 1979) and sown on nutrient agar containing Parker's macronutrients and Thompson's micronutrients (Klekowski, 1969). The resulting gametophtyes were grown for fifteen weeks at a mean temperature of 19.6°C ( $\pm$  0.64) and under a bank of grow lights with a mean light level of  $27.0/m^2/sec. (\pm 2.8)$  and a sixteen hours light : eight hours dark regimen. At the end of fifteen weeks, the gametophytes were discarded and an extract from the agar was obtained by a freeze-thaw process. Suspended matter was removed from the extract by centrifugation and the supernatant was diluted by fifty-percent using Parker-Thompson's nutrients. Fresh agar was then added and the resulting solution was used to make a treatment agar. In the first experiment, over one hundred three-week old cordate gametophytes (i.e., exhibiting conspicuous apical notches) were transferred to petriplates containing either treatment agar or a control containing basal nutrient agar (Figure 1). No antheridia or archegonia were observed in cordate gametophytes at the time of transfer. Gametophytes were evenly spaced at

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FIG. 1. Gametophyte silhouettes illustrating relative sizes and shapes at beginning and end of second experiment: (a) six-week old gametophyte at time of transfer, (b) largest gametophyte in control population and (c) largest gametophyte in treatment population at end of experiment. Shape factor (SF) is listed immediately below each gametophyte and arrows within in each gametophyte illustrate linear measurements. Gametophyte length was measured from the base of a gametophyte to its notch (solid white arrow) and width was measured as the longest line (dashed white arrow) perpendicular to the length line. Notch depth (solid black arrow) was measured as an extension of the length line from the deepest point in the notch to the line connecting the most distal points of the apical lobes (dashed line).

a density of approximately one gametophyte per square centimeter. Petri-plate lids remained unsealed to facilitate gaseous exchange and reduce ethylene buildup. Twenty-one days after transfer, fifty gametophytes per control and treatment were harvested and mounted on microscope slides using permount. In a second experiment, the entire procedure was repeated using six-week old cordate gametophytes (Figure 1a) at time of transfer and harvesting fifty-five gametophytes per control and treatment. Archegonia in various states of development were observed on all individuals at time of transfer in this experiment. No antheridia were observed.

Each gametophyte was photographed at  $8.5 \times$  using a digital camera attached to a dissection microscope. Gametophyte photographs were magnified  $2 \times$  on a computer and size (area in pixels) and shape (circularity) of each gametophyte was measured using Sigmascan Pro 4.0 (Fox and Urich, 1993). Circularity was analyzed using the shape factor function and was calculated using the formula  $(4\pi \times \text{Area})$  / Perimeter<sup>2</sup>. Shape factor values range from zero to one, indicating linearity and circularity, respectively. In addition to size and shape, gametophyte length, width and notch depth were also measured in the second experiment using the linear distance function in Sigmascan Pro 4.0. Gametophyte length was measured from the base of the gametophyte to its notch (solid white arrow) and width was measured as the longest line (dashed white arrow) perpendicular to the length line (Figure 1). Notch depth (solid black arrow) was measured as an extension of the length line from the deepest point in the notch

TABLE 1. Equal variance t-tests and Mann-Whitney U rank sum tests of size and shape, and chisquare tests of frequency of antheridia-bearing individuals and archegonia per gametophyte, of three-week old and six-week old *A. felix-femina* gametophytes transferred to basal nutrient agar (control) versus a nutrient agar that supported a previous generation of gametophytes (treatment). Shape factor values approaching zero indicate a linear morphology, whereas those approaching one indicate a circular morphology.

Trait	Control	Treatment	Statistic, P-value
Three-week old gametophyte	s (at transfer)		
# of Gametophytes	50	50	
Size (cm <sup>2</sup> )*	$1.10 \pm 0.46 \text{ cm}^2$	$0.71 \pm 0.36 \text{ cm}^2$	T = 5.01, P < 0.001
Shape factor	$0.28 \pm 0.18$	$0.26 \pm 0.18$	U = 1.16, P = 0.123
Antheridial gametophytes/			
population	5	19	$X^2 = 10.44$ ; P < 0.005
Archegonia/population			
(per gametophyte)	759 (14.3 ± 9.3)	405 (7.6 ± 7.2)	$X^2 = 109.38; P \le 0.001$
Six-week old gametophytes (a	at transfer)		
# of Gametophytes	55	55	
Size (cm <sup>2</sup> )*	$1.09 \pm 0.32 \text{ cm}^2$	$1.02 \pm 0.23 \text{ cm}^2$	T = 1.35 P = 0.090
Shape factor	$0.40 \pm 0.24$	$0.49 \pm 0.22$	II = -2.44 P = 0.007
Length	$76.8 \pm 12.7$	$75.4 \pm 12.11$	T = 0.51 P = 0.30
Width	$152.0 \pm 19.2$	$165.8 \pm 31.3$	II = 1980 P = 0.006
Length : width ratio	$0.51 \pm 0.09$	$0.47 \pm 0.02$	T = 1.98 P = 0.03
Notch depth	$31.2 \pm 7.6$	$37.6 \pm 10.2$	T = 3.35 P < 0.001
Antheridial gametophytes/			
population	0	1	
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Archogonia/nonulation

ruchegoma/population			
(per gametophyte)	1688 (33.1 ± 13.2)	1461 (28.7 $\pm$ 11.5)	$X^2 = 16.38, P < 0.001$

\* Converted from image area in pixels to reflect true size.

to the line connecting the most distal points of the apical lobes (dashed line, Figure 1). Numbers of archegonia and antheridia on each gametophyte were counted directly using a compound microscope in both experiments.

Size and shape data were analyzed using equal variance t-tests, except when data failed to meet assumptions of normality and homoscedasticty, in which case Mann-Whitney U rank sum tests were performed. Data sets were tested for normality using Pearson-D'Agostino omnibus tests and for homoscedasticity using modified-Levene tests. A critical value of P = 0.05 was used for all statistical procedures. All statistical analyses were conducted using NCSS 97 (Hintze, 1997).

### RESULTS

In the first experiment, individuals from the treatment population were not significantly different in shape, but were significantly smaller, possessed fewer archegonia, and contained more antheridial individuals (both as males and bisexuals), than individuals from the control population (Table 1). Thus, at three-weeks from germination, 38% of cordate gametophytes were still able to respond to antheridiogen by producing antheridia.

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In the second experiment, in which six-week old gametophytes were transferred, the treatment population was more circular, possessed greater widths, smaller length : width ratios and deeper notches than the control population (Table 1). Collectively, these observations indicate that lateral growth generated by the apical meristem was greater in the treatment population than in the control population. The treatment population also possessed significantly fewer archegonia (13.3%) than the control population (Table 1). Differences in length and the proportion of males in each population were not significant (Table 1); however, the marginal P-value (0.09) associated with the t-test for difference in size (Table 1) may indicate a weak negative treatment effect. No antheridia were found in the control population and only one gametophyte (1.8%) possessed antheridia in the treatment population.

#### DISCUSSION

Pheromonal interactions between cordate and acordate gametophtyes, mediated through antheridiogen secretion by the former and uptake by the latter, are known for many filicalean ferns (Näf et al., 1975; Raghavan, 1989). Cordate gametophytes undoubtedly release numerous other substances into their surroundings that may include water-soluble regulators of their own growth and production of gametangia. These substances may influence neighboring cordate gametophytes. Such interactions may provide insight into the phytochemical regulation of gametophyte development and reproductive

ecology.

Results from our second experiment demonstrate that cordate gametophytes of *A. filix-femina* produce one or more water-soluble substances that accelerate lateral growth, and subsequent development of a circular profile and deeply recessed notch meristem, and retard production of archegonia in cordate gametophytes of the same species. The ecological relevance of these cordatecordate interactions remains unclear, however, it is not difficult to envision a fitness advantage to reducing the risk of polyembrony in dense populations with a high likelihood of fertilization success.

Concentrations of the phytochemicals used in this study are unlikely to occur in nature. Although the treatment agar was diluted by fifty percent, it represented the accumulation of fifteen weeks of water-soluble metabolites. In the wild, drainage and biotic and abiotic interactions within soil probably reduced concentrations of these phytochemicals well below those used here. Nevertheless, Greer and McCarthy (1997) observed a peak in cordate males in populations growing on soil at the periphery of the antheridiogen neighborhood (the horizontal range of effect from a source gametophyte) of *Polystichum acrostichoides* (Michx.) Schott. Thus, the substance(s) responsible for the effects observed in this study may reach sufficient levels in nature to induce the responses we observed. The seemingly contradictory effects of increased circularity without increased size may be the result of one or more water-soluble hormones involved in gametophyte morphogenesis. Morphological development in most filicalean

gametophytes is a function of the planes of division occurring in the meristem (Raghavan 1989). Transition from a one-dimensional filamentous morphology to a two-dimensional spathulate morphology involves the initiation of oblique and anticlinal divisions, as opposed to periclinal divisions, in the single-celled meristem (von Aderkas and Cutter, 1983; Raghavan, 1989). Likewise, transition from a spathulate to a cordate morphology results from an increase in anticlinal divisions in the meristem and its derivatives (von Aderkas and Cutter, 1983; Raghavan, 1989). In cordate gametophytes, anticlinal divisions in the meristem tend to produce small, columnar cells, whereas periclinal and oblique divisions tend to produce larger cells that have a greater impact on overall size (von Aderkas and Cutter, 1983). Thus, the presence of one or more phytochemicals in the treatment may have accelerated anticlinal divisions at the expense of oblique or periclinal divisions, resulting in a deeper notch and wider, more circular profile without a corresponding increase in size. This may also explain the decreased production of archegonia in the treatment in the experiment using six-week old gametophytes, because archegonia are ultimately derived from oblique and periclinal divisions of the meristem. Although the identity of the substance or substances inducing the effects observed in this study remain unknown, antheridiogen is a candidate. Antheridiogen is a stable, water-soluble compound produced by cordate gametophytes that overrides the light requirement for spore germination, induces precocious maleness in filamentous and spathulate gametophytes, and subsequently retards growth and morphological development in acordate neighbors (Näf et al., 1975; Raghavan, 1989). Structurally, antheridiogen is similar to gibberellin (Näf et al., 1975; Yamauchi et al., 1996; Nester-Hudson et al., 1998), which has similar effects on many seed plants; i.e., it stimulates seed germination and induces maleness in flowers. Recognizing the structural and functional similarity between many known antheridiogens and gibberellin, and the ability of gibberellins to substitute for the antheridiogen of some species of ferns (Näf et al., 1975; Raghavan, 1989), a few authors have speculated about a hormonal role for antheridiogen (Willson, 1981; Voeller and Weinberg, 1969; Schraudolf, 1985; Greer and McCarthy, 1997). Greer and McCarthy (1997) advanced two hypotheses that may be relevant here, the hormone-pheromone and multiple-signal hypotheses. The hormonepheromone hypothesis suggests that antheridiogen is a hormone involved in the development and persistence of a cordate morphology in a source gametophyte. In addition to the similarities between antheridiogen and gibberellin listed above, this hypothesis emphasizes the correspondence between the development of a cordate morphology and the production of antheridiogen, and the ability of gibberellin to stimulate cell division in the shoot apex of seed plants. According to the hormone-pheromone hypothesis, response to antheridiogen changes following attainment of a cordate morphology from production of antheridia, reduced growth, and delayed attainment of circularity, to accelerated attainment of circularity and reduced production of archegonia. This model may explain the inhibitory effect of GA<sub>3</sub> on archegonial development in Lygodium japonicum (Takeno et al., 1979) and prolonged production of

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antheridia and delayed production of archegonia by cordate gametophytes of *Onoclea sensibilis* exposed to antheridiogen of *Pteridium aquilinum* beginning during acordate stages (Näf et al., 1975). It is noteworthy here that genes (TRA and MAN) that control gender expression and are indirectly responsive to antheridiogen in gametophytes of *Ceratopteris richardii* appear to also regulate sporophyte development in this species (Banks 1997).

Alternatively, the multiple-signal hypothesis suggests that cordate gametophytes release two or more water-soluble pheromones. If a compound other than antheridiogen is responsible for accelerated attainment of a circular profile, it has no apparent effect on acordate gametophytes. Under this scenario, receptivity to pheromonal influences on gametophyte development gradually shifts from antheridiogen during acordate phases to one or more other pheromones that affect cordate gametophytes. In conclusion, cordate gametophytes of *Athyrium filix-femina* produce a water-soluble compound that accelerates the development of a circular profile and retards production of archegonia in well-developed cordate gametophytes. The identity of the substance eliciting these effects remains unknown, however antheridiogen or another gibberellin like substance is a possibility. To answer the many questions raised by this study, similar experiments need to be conducted using isolated antheridiogen and other water-soluble substances released by fern gametophytes.

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